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The Protective Effect of Melanin Against Radiation

Final Technical Report

By Patricia A. Baldry and George A. Swan

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20. ABSTRACT:

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The ability of melanin to catalyse the in vitro oxidation of reduced nicotinamide-adenine dinucleotide was shown to be independent of the melanin's free radical properties and an electron transfer mechanism was proposed. Melanin did not catalyse the oxidation of 3,4-dihydroxyphenylalanine showing that melanin does not catalyse its own formation.

Oxidation and reduction of autoxidative dopa-melanin were studied. Reduction with neither ascorbic acid nor sodium dithionite led to any overall increase in the number of 5,6-dihydroxyindole units in the melanin polymer. The melanin was oxidised using respectivley potassium ferricyanide and ceric sulphate solutions but in neither case was the melanin e.s.r. signal diminished.

The interaction of melanin's free radicals with cupric ions was shown not to be chemical in nature. The number of bound cupric ions was determined and the value used to calculate the number of free radicals in autoxidative dopa-melanin.

SUMMARY

The electron transfer and free radical properties of melanins were studied to discover whether melanin might protect the living cell against toxic free radicals produced for example by solar irradiation. The effects of some stable radicals on the e.s.r. signal of autoxidative dopa-melanin suggested that direct reaction between melanin free radicals and other radicals was unlikely.

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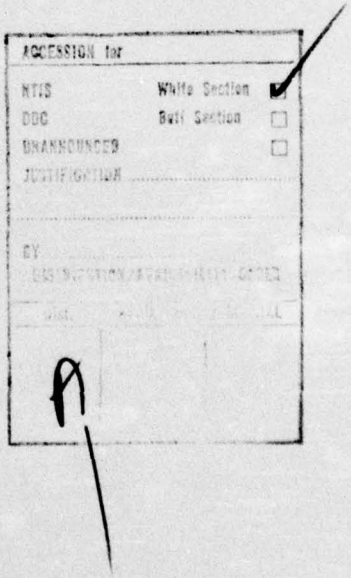

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INTRODUCTION

Melanins are irregular polymers derived biosynthetically from tyrosine, dopa and possibly dopamine and which occur in human skin, retinal pigment, hair, wool, feather, the skin of reptiles and fish, the ink sac of cephalopods, the liver of axolotls, the pineal gland, malignant melanoma, and possibly the brain of man and monkeys (Substantia nigra). Current views on the structure, chemistry and biosynthesis of melanins are reviewed by Swan.[1] In 1928 Raper[2] suggested that melanins were polymers of indole-5,6-quinone derived via oxidation of tyrosine through dopa, dopaquinone, dopachrome and 5,6-dihydroxyindole. This is now believed to be an oversimplification. Other compounds of suitable reactivity (particularly phenolic compounds, or other intermediates in the Raper Scheme) may be incorporated into the polymer; and subsequently some of the polymer units may undergo structural changes owing to oxidation.

In 1954 Commoner et.al.[3] detected electron paramagnetic resonance signals in natural melanin and proposed that the paramagnetism was due to free radicals trapped in the pigment. The trapping of free radicals by a growing polymer has been demonstrated in the case of polymethacrylate.[4]

The electron spin resonance spectra of melanins are generally characterised by single, usually structureless, absorption lines, g-values near 2 and a paramagnetism that is permanent. Blois[5] compared melanin g-values with those from aromatic radicals of known structure and concluded that the unpaired electron is delocalized over one or at most two aromatic rings.

It has been suggested[6] that melanins might participate in certain biological redox reactions and it was shown that melanins catalyse the in vitro oxidation of reduced nicotinamide-adenine dinucleotide. The catalytic activity was inhibited by pharmacologically active compounds that combine with melanin such as chlorpromazine and chloroquine.

In addition it has been suggested[7] that melanin, having free-radical properties, might protect the living cell against toxic free radicals (e.g. formed by radiation). Little is yet known about these properties of melanins and of their possible biological function.

The aim of the present work was to investigate the electron transfer and free radical properties of melanins.

RESULTS AND DISCUSSION

It was suggested[6] that the ability of melanin to catalyse the *in vitro* oxidation of β-NADH was related to melanin's free-radical properties. Blois[5] however has shown that the radicals in melanin are extremely stable. The e.s.r. signal was unaffected by treatment of the melanin with acid or alkali or ascorbic acid. He attributed this stability to the radical being buried deeply in the three-dimensional polymer.

An alternative explanation to a radical mechanism might be that oxidation of β-NADH occurs *via* an electron transfer process. Quinone units in the melanin might act as electron acceptors, subsequent reoxidation of the reduced melanin being achieved by molecular oxygen.

In the present work it was shown that the melanin-catalysed oxidation of β-NADH was independent of the melanin's free radical properties. The approach used was based on the known[5] ability of cupric ions to quench the paramagnetism of the melanins. A sample of autoxidative dopa-melanin was divided into two equal batches one of which was treated with excess of cupric ions. Residual copper was removed by rigorous washing with 2N-hydrochloric acid. The cupric ion treated melanin showed no e.s.r. signal in contrast to the control batch which showed the characteristic melanin resonance.

The catalytic abilities of the two samples were compared. The oxidation of β-NADH was followed manometrically in the Warburg[29] apparatus at 37° and pH 8.0. The rate of oxygen consumption was the same for both samples indicating that the oxidation was not catalysed by the free radicals within the melanin polymer. This was consistent with the known[5] stability of melanin free radicals.

Further evidence against a free radical mechanism was the observation that the e.s.r. signal amplitude of autoxidative dopa-melanin was not changed after prolonged treatment with β-NADH at pH 8.0.

The phenothiazines, *e.g.* trifluoperazine, chlorpromazine, promethazine and ethopromazine inhibit the ability of melanin to catalyse the oxidation of β-NaDH. These compounds are known[8,9] to bind preferentially to melanin-bearing tissues and a possible explanation might be that bound phenothiazine molecules block the reaction centres of the melanin polymer.

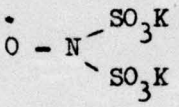
Van Woert[6] suggested that treatment with the phenothiazine might result in a reduction in the number of free radicals in the melanin. This explanation is less likely as the radicals have been shown to be extremely stable. The same argument might be raised against the findings of Forrest *et.al.*[10] who reported that chlorpromazine treatment reduces the free radical signal of melanin by approximately 50%.

In the present work autoxidative dopa-melanin was stirred with a solution of chlorpromazine at pH 5.0 for 20 hours. The melanin was collected, dried and the amount of bound chlorpromazine was determined. The phenothiazine was extracted into ethanol and its concentration determined by a u.v. method. The melanin was found to bind its own weight of chlorpromazine.

The e.s.r. spectrum of the chlorpromazine-treated sample (corrected for the weight of bound chlorpromazine) was compared with that of a control sample. The signal amplitudes were the same showing that treatment with chlorpromazine resulted in no reduction in the number of melanin free radicals.

It has been suggested that melanin having free-radical properties, might act as a "free radical trap" and protect the living cell against toxic free radicals produced for example by solar irradiation. Little however is yet known about these radical scavenging properties of melanins. It has been reported[11] that the diphenylpicrylhydrazyl (DPPH) radical reacts with melanin in a 50% ethanolic pH 5.5 phosphate-citrate buffer. The reaction was shown[11] not to be a radical-radical reaction but instead a simple hydrogen abstraction by DPPH to form the diphenylpicrylhydrazine.

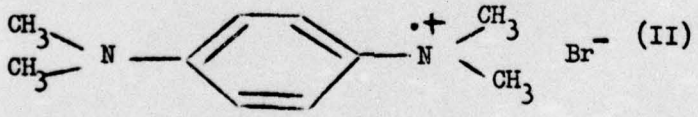
Smaller free radicals might be able to penetrate to the radical-containing regions of the polymer and react with the melanin free radicals. The effect of the small Frémy radical (I) was studied.



(I)

Autoxidative dopa-melanin was stirred for 2 hours with an alkaline (pH 9.0) solution of (I). Excess of Frémy radicals were decomposed by treatment with 2N-hydrochloric acid[12] and the melanin was collected. The e.s.r. signal amplitude was identical with that of a control sample that had not been treated with (I). This showed that a radical-radical reaction had not taken place.

Similarly, no change in e.s.r. signal intensity was observed when autoxidative dopa-melanin was treated with an acidic solution of Wursters blue, radical-cation (II).



(II)

These results substantiate the trapped free radical hypothesis and suggest that direct reaction with other radicals (e.g. toxic radicals formed by radiolysis of compounds present in the organism) is unlikely.

In 1942 Rothman[13] reported that melanin obtained from the ink sac of the squid or produced by the autoxidation of dopa was lightened in colour from jet black to light tan by ascorbic acid. More recently Blois[5] showed that the e.s.r. signal of squid melanin was unaffected by mild treatment with excess of ascorbic acid. The melanin turned dark brown indicating that melanin colour and paramagnetism are independent of each other.

In direct contrast Mason[7] reported that the e.s.r. absorption of Sepia - melanin was halved in intensity after reduction with excess of ascorbic acid. Further conflicting evidence was presented by Chauffe, Windle and Friedman.[14] They reported a 64% decrease in e.s.r. signal intensity after heating wool melanin under reflux overnight with 2M-ascorbic acid.

In all cases natural melanin samples were used which may or may not have contained admixed protein together with various paramagnetic ions (e.g. Cu^{2+}, Fe^{3+} or Mn^{2+}). Trace cupric ions catalyse the oxidation of ascorbic acid at pH values less than 7.6.[15] In addition ascorbic acid is rapidly decomposed when heated. These decomposition reactions might interfere with the melanin-ascorbic acid reaction and lead to erroneous conclusions.

The effect of mild ascorbic acid treatment on the physical and chemical properties of autoxidative dopa-melanin was investigated. This synthetic melanin contains no paramagnetic ions or admixed protein and would not be subject to the above side-reactions. The melanin was stirred at room temperature for a) 21 hours and b) 5 days with excess of ascorbic acid at pH 9.0. No change in e.s.r. signal intensity was found in either case. In addition the reported[7,14] colour change was not observed. Rapid aerial oxidation of reduced melanin was ruled out as the melanin was not lightened in colour when the reduction was carried out under nitrogen.

Colour change observations having proved inconclusive recourse was made to alternative methods for studying the course of the ascorbic acid reduction of melanin. Reduction of indole-5,6-quinone units would result in an increase in the number of 5,6-dihydroxyindole units and this might be determined by methoxy-analysis after methylation with diazomethane. Treatment of melanins with diazomethane results in the methylation of carboxy- and phenolic groups to give methoxy-groups which are determined by the Zeisel method. An increase in methoxy-value after ascorbic acid treatment would indicate that reduction had taken place.

A sample of autoxidative dopa-melanin was divided into two equal batches and one batch was stirred with ascorbic acid at pH 9.0 for 2 days. Both samples were methylated exhaustively with diazomethane and their methoxy- values were compared. The ascorbic acid treated sample value (19.3%) differed only slightly from that of the control (18.01%). It was thus concluded that reduction had not taken place to a significant extent.

The possibility that melanin might catalyse the autoxidation of ascorbic acid without itself undergoing any permanent physical or chemical change was investigated. Ascorbic acid is a weaker acid ($K_1 = 2 \times 10^{-5}$; $K_2 = 6 \times 10^{-13}$) than dehydroascorbic acid ($K_1 = 6 \times 10^{-4}$; $K_2 = 1.2 \times 10^{-8}$) and oxidation proceeds with a decrease in pH. The rate of decrease in pH was used to monitor the rate of autoxidation of ascorbic acid in respectively the presence and absence of autoxidative dopa-melanin. The rates were found to be identical showing that melanin does not catalyse the oxidation of ascorbic acid.

Experiments were carried out to ascertain whether treatment with other reducing agents might bring about a pronounced lightening in colour of the melanin pigment. In 1939 Figge[16] reported that melanins are turned light tan on treatment with sodium dithionite but no experimental details were given. In the present work autoxidative dopa-melanin was stirred for 5 hours with a sodium dithionite solution at pH 11.0. Both the melanin colour and e.s.r. signal intensity were unchanged after this treatment.

The sample was exhaustively methylated with diazomethane and methoxy- values were determined as described above. The value found (18.2%) was extremely close to that of a control sample (18.0%) and showed that no overall increase in the number of 5,6-dihydroxyindole units had taken place.

Autoxidative dopa-melanin was however appreciably lightened in colour when the sodium dithionite reduction was carried out under nitrogen. The light brown coloured melanin was precipitated under nitrogen by the addition of 2N-hydrochloric acid. The sample darkened on centrifugation and after drying *in vacuo* over phosphoric oxide for 3 days was jet black. A possible explanation might be that after removal of the solvent rapid aerial oxidation of the reduced melanin took place.

Similar observations were reported by Das, Abramson and Katzman.[17] They found that reduction of melanins with sodium borohydride yielded clear, pale brown solutions that were stable for several months at -60°. The solutions were unstable at room temperature and darkened substantially on drying.

If indeed the free radicals in melanin are extremely stable a number of questions remain unanswered. Blois[5] reported that the e.s.r. signal intensity of squid-melanin was dependent upon the state of hydration of the sample. Upon drying, the signal amplitude increased to a maximum of six times its original intensity and then decreased to about twice the initial value. The e.s.r. signal was not eliminated when the melanin was heated in oxygen at 200-500°. The signal was however destroyed when the melanin was treated with a sufficiently high concentration of cupric ions. Blois suggested that the small cupric ions are able to penetrate to the deeply buried free radical sites. The question as to why cupric ions and water molecules, but not oxygen molecules, penetrate to the radical sites was unanswered.

Melanins are hygroscopic and can bind up to 30% their own weight of water.[5] The effect of hydration on the e.s.r. signal of autoxidative dopa-melanin was studied. A melanin slurry was stored for 20 hours *in vacuo* over calcium chloride. The partly dried melanin was divided into two equal batches. One batch (hydrated sample) was weighed into an e.s.r. tube and the tube was immediately sealed. The second batch (dehydrated sample) was dried to constant weight *in vacuo* over phosphoric oxide. The measured weight loss showed the hydrated sample to contain 17% by weight of water.

The e.s.r. signal intensities of the two batches were identical indicating that the free radicals were not destroyed. In contrast to the spectra of Blois the above signals showed no saturation effects over the range of incident microwave power used. This would confirm Blois'[5] suggestion that the decrease in signal intensity he observed was due simply to a variation in spin-lattice relaxation time and not to a chemical reaction between bound water molecules and the melanin free radicals.

Experiments were carried out to discover whether the observed[5] interaction of cupric ions with the melanin free radicals was of a chemical nature. Cupric ions might penetrate to the radical sites and oxidise the buried radicals by an electron transfer reaction. To test this hypothesis the effects of other oxidising agents were investigated. In 1939 Figge[16] reported that melanins reduced by sodium dithionite were re-oxidised by potassium ferricyanide. More recent kinetic studies by Gan, Haberman and Menon[18] showed that melanins are rapidly oxidised by potassium ferricyanide.

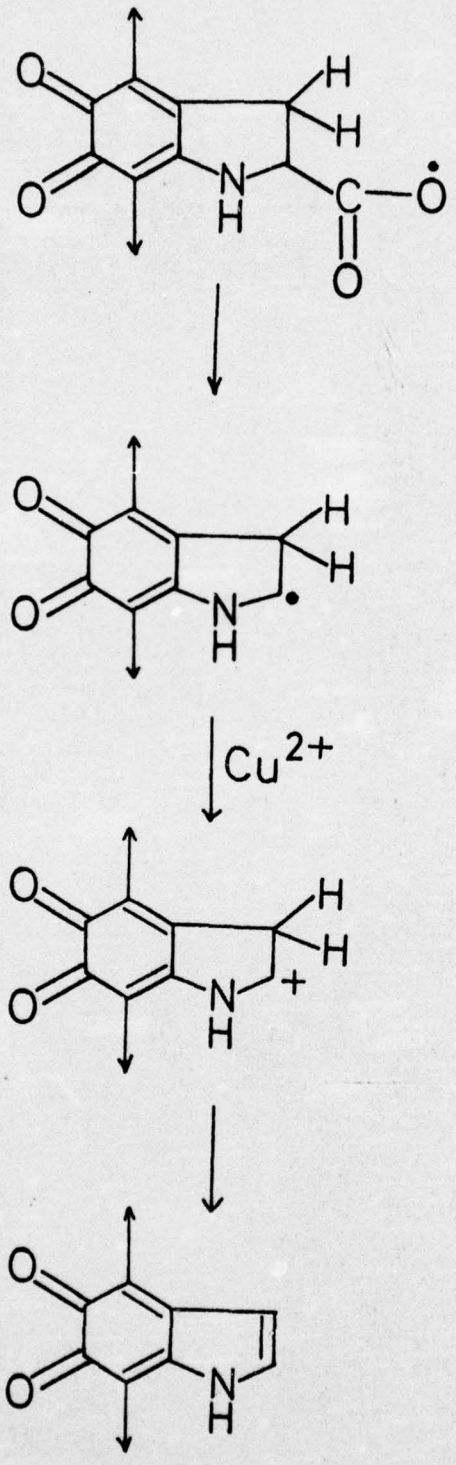
Autoxidative dopa-melanin was stirred with a solution of potassium ferricyanide for twenty minutes at pH 10. The e.s.r. signal intensity was unchanged showing that treatment with potassium ferricyanide does not destroy the melanin free radicals.

No change in e.s.r. signal intensity was also found when the melanin was stirred for five minutes with a ceric sulphate solution at pH 3. The resulting melanin was warmed (60-70°) with 3.6 N-sulphuric acid to remove bound cerium cations. Cerous ions were extracted and their concentration (28% by weight) was determined by u.v. spectroscopy. The strongly oxidising ceric cations were thus shown to be reduced to Ce^{3+} by the melanin without the melanin e.s.r. signals being affected.

The above results suggested that a cupric ion catalysed oxidation of the melanin polymer was unlikely. An alternative explanation might be that cupric ions catalyse decarboxylation reactions e.g. (III) ⟶ (IV) showed in FIGURE 1.

This was shown not to be the case as the e.s.r. signal of autoxidative dopa-melanin was not eliminated when the sample was decarboxylated (230°, 0.1 mm Hg, 12 h).

Zinc salts are known[19] to catalyse the rearrangement of aminochromes to 5,6-dihydroxyindoles and by analogy might catalyse the reaction (III) ⟶ (IV). Lukiewicz[20] studied the effect of zinc ions on the e.s.r. signal amplitude of melanin pigment from damaged amphibian eggs and embryos. The results were difficult to interpret and different melanin preparations gave widely different responses. Lukiewicz postulated that the observed response was dependent on the number of paramagnetic ions in the various melanin preparations. Natural melanins contain paramagnetic ions (e.g. Cu^{2+}, Fe^{3+} or Mn^{2+}) that partly quench the melanin free radical resonance. Replacement of these ions by diamagnetic zinc ions would be expected to increase the free radical signal.



(III)

(IV)

FIGURE 1

Autoxidative dopa-melanin, unlike the natural melanins contains no paramagnetic ions. Treatment of this melanin with zinc ions might be expected to show the true effect of the ions on the melanin free radicals. The melanin was stirred for five minutes with zinc sulphate solution. The e.s.r. signal intensity was unchanged after this treatment showing that zinc ions do not react with the melanin radicals.

The above result confirmed Lukiewicz' finding that zinc ions have no effect on the e.s.r. signals of melanin samples that have been boiled with concentrated hydrochloric acid and paramagnetic ions thus removed.

The above results indicated that the interaction between melanin free radicals and cupric ions was not chemical in nature and further confirmed the extreme stability of the radicals. These conclusions were consistent with the recent work of Sarna *et.al.*[21] who showed that the apparent reaction of melanin free radicals with paramagnetic copper was a purely physical consequence of magnetic dipolar interaction. Paramagnetic lanthanide ions *e.g.* Gd^{3+} eliminated the e.s.r. signal of bovine choroid-melanin whilst chemically similar diamagnetic ions *e.g.* La^{3+} were without effect. Competition experiments between diamagnetic and paramagnetic metal ions for melanin binding sites indicated the presence of several distinct types of binding sites.

The melanin samples studies by Swartz[21] were isolated by a mild extraction procedure that left protein still incorporated in the granules. His results are therefore subject to two limitations. The true melanin e.s.r. signal might be partly quenched by paramagnetic contaminations accompanying the non-hydrolysed peptide remnants. Secondly metallic ions might preferentially bind to protein and thus lead to erroneous conclusions about the true melanin binding sites.

A study of the metallic ion binding properties of autoxidative dopa-melanin might give a better indication of the nature and number of the melanin's binding sites. This melanin contains neither admixed protein nor paramagnetic ions and would not therefore be subject to the above limitations.

The e.s.r. signal of autoxidative dopa-melanin was eliminated after the melanin was stirred for five minutes with copper sulphate solution. In a second experiment the copper sulphate treated melanin was washed three times with 2N-hydrochloric acid. The e.s.r. signal was not restored indicating that bound cupric ions were not removed by acid washing.

Autoxidative dopa-melanin was treated with excess of cupric ions and the amount of bound cupric ions was quantatively determined. The initial approach was to remove the bound cupric ions as their yellow-brown, sparingly soluble sodium diethyldithiocarbamate complex,[22] to extract this complex into carbon tetrachloride and to determine the cupric ion concentration colorimetrically against standard solutions. The procedure was unsuccessful as the tightly bound cupric ions were not removed by the complexing reagent.

In a modified procedure the melanin was boiled with concentrated nitric acid for three hours. The pale yellow solution was evaporated to dryness and the residue was treated with sodium diethyldithiocarbamate solution. The copper diethyldithiocarbamate complex was determined colorimetrically as described above. The result showed that 90 mg of autoxidative dopa-melanin bound 0.75 mg of cupric ions after treatment with an excess of cupric ions (12.8 mg).

A similar result was obtained using a melanin preparation that was washed four times with N-hydrochloric acid after treatment with copper sulphate solution. 70 mg of the melanin bound 0.5 mg of cupric ions confirming that bound cupric ions were not removed by acid washing.

Little is known about the nature and number of melanin-cupric ion binding sites. In addition the relative number of binding sites to free radicals is not known. If one assumes a monomer molecular weight of 147 then 0.75 mg of bound cupric ions per 90 mg of melanin is equivalent to one bound cupric ion per 50 units of monomer. Autoxidative dopa-melanin therefore contains relatively few cupric ion binding sites. This synthetic melanin contains no admixed protein and it is possible that protein present in natural melanins might provide additional binding sites.

Other aspects of melanogenesis were investigated. These included a study of the effect of pH on the rate of oxidation of dopa. Oxidation of dopa to melanin is usually achieved by the enzyme tyrosinase at pH 6.8; although under alkaline conditions (even at pH 8.0) this can be achieved by autoxidation. It was of interest to ascertain whether the oxidation of dopa at pH 6.8 could be achieved in the absence of enzyme but presence of melanin i.e. that melanin might catalyse its own formation.

The rate of oxygen uptake during the conversion of dopa into melanin was followed manometrically in the Warburg apparatus at 37°. The autoxidation of dopa at pH 6.8 was found to proceed very slowly after a long induction period. Identical results were obtained using respectively 0.1 M-phosphate and 0.53 M-citrate buffers showing that both the rate of oxidation and the induction period were independent of the buffer solution used. The oxidation was not catalysed by autoxidative dopa-melanin and it was therefore concluded that melanin does not catalyse its own formation at pH 6.8.

Under alkaline conditions at pH 8.0 the autoxidation of dopa was more rapid. The results were not reproducible and on separate occasions appeared to fall into one of two types. The slower reaction required 4 atoms of oxygen per molecule of dopa oxidised whilst the faster process required 7.4 atoms of oxygen per mol. of dopa. These values are to be compared with the results of Mason and Wright.[23] They found that the enzymic conversion of dopa into melanin required between 2.9 and 4.6 atoms of oxygen per mol. of dopa oxidised. The exact values depended on numerous factors such as pH, temperature and enzyme purity.

The quantities of hydrogen peroxide produced during the two types of oxidation were determined by precipitating the melanin with 3.6 N-sulphuric acid and titrating the clear supernatant with 0.1N-potassium permanganate solution. The slower reaction produced 1.3 mol. and the faster reaction 2.7 mol. of hydrogen peroxide. Hydrogen peroxide decomposition was ruled out as "20 volume" hydrogen peroxide was shown manometrically to be stable at 37° and pH 8.0.

The above results suggested that the oxidation of dopa in phosphate buffer pH 8.0 might proceed *via* two different mechanisms. The results however were not reproducible and definite conclusions could not be drawn.

The enzymic preparation of dopa-melanin was investigated. Binns *et.al.*[24] studied the oxidation of dopa at pH 6.8 and 20° in the presence of mushroom polyphenol oxidase. The resulting melanin was not centrifugable (300,000 *g*) and was precipitated with 2N-hydrochloric acid. In contrast Van Woert reported[25] the preparation of insoluble enzymic dopa-melanin that was readily centrifuged. In this preparation dopa was oxidised at pH 6.8 and 37° in the presence of purified mushroom tyrosinase.

The above reactions were further investigated to ascertain the factors that determine the solubility properties of enzymic dopa-melanin. In two separate experiments dopa was oxidised with mushroom tyrosinase at pH 6.8 for 24 hours. The first experiment was carried out at 37° and the second at 19°. Melanogenesis was rapid at 37°, the solution became red almost immediately and the melanin was readily centrifuged (1,500 *g*, 5 min). At 19° oxidation was much slower and the resulting melanin was not centrifugable. The solubility properties of enzymic dopa-melanin therefore depend on the temperature used for its preparation.

The above preparations were carried out in buffered solutions at pH 6.8. This approach suffers the limitation that the melanin might absorb inorganic ions (*e.g.* Na^+ and K^+) from the buffer solution. Mason, Ingram and Allen[7] reported the preparation of enzymic dopa-melanin in 30% yield in distilled water as solvent.

This result was surprising as the inherent acidity of the distilled water might be expected to prevent the cyclisation of dopaquinone to leucodopachrome and thus inhibit melanogenesis. Attempts to repeat the preparation were unsuccessful. Oxidation of dopa in triply-distilled water in the presence of mushroom tyrosinase gave very low yields (3 - 5%) of melanin. The final solutions were acidic (pH 3 - 4.5) and this acidity probably prevented further melanin formation.

Harley-Mason[26] reported that the oxidation of 6-methyldopa (V) with mushroom tyrosinase at pH 6.85 gave the product (VI) as a stable yellow solution (see Figure 2). All attempts to isolate any solid product or bring about a rearrangement to an indole were unsuccessful.

In the present work the autoxidation of 6-methyldopa at pH 8.0 was studied.

6-Methyldopa rapidly took up one atom of oxygen at pH 8.0 to give a stable, yellow solution. The u.v. spectrum was different to that reported[26] and resembled the spectrum of the starting material. The u.v. chromophore was unchanged after nitrogen gas was bubbled through the solution for 18 h. This ruled out the formation of a weak molecular complex between oxygen and 6-methyldopa (*c.f.* the yellow complex obtained[27] by the oxidation of NN-dimethylaniline).

E.s.r. studies by Wertz *et.al.*[28] indicate that some oxidation of the dopa side-chain takes place during the autoxidation of dopa to melanin. A possible explanation of the above results thus might be that oxygen uptake by 6-methyldopa results in the oxidation of the side-chain.

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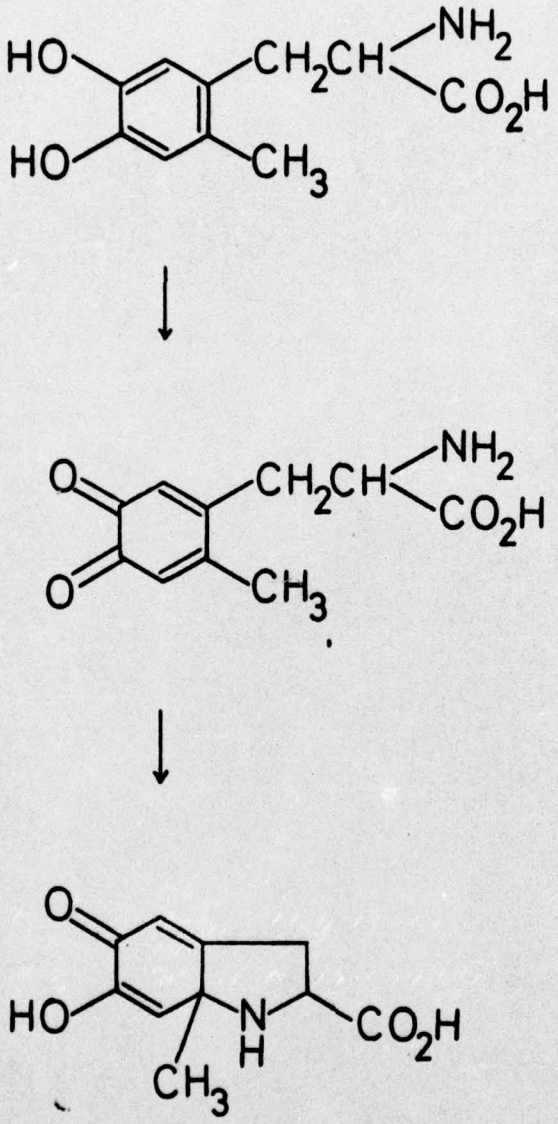

(V)

(VI)

FIGURE 2

EXPERIMENTAL

Preparation of Autoxidative Dopa-Melanin. - (±)-3,4-Dihydroxyphenylalanine (125 mg) was dissolved in 0.1 M-phosphate buffer of pH 8.0 (50 ml). Oxygen gas was passed for 18 h through this solution at 37°. The melanin was precipitated with 2N-hydrochloric acid (5 ml), centrifuged, washed with water (10 ml) and dried *in vacuo* over phosphoric oxide; yield *ca.* 60 mg.

Oxidation of Reduced Nicotinamide-Adenine Dinucleotide (β-NADH) was studied manometrically by the Warburg apparatus[29] at 37°. The main compartment of each flask contained autoxidative dopa-melanin (15 mg) suspended in 0.1 M-phosphate buffer pH 8.0 (4.0 ml). The side arm contained β-NADH (10.3 mg) in 0.1 M-phosphate buffer pH 8.0 (0.8 ml). The inner cup contained 10% sodium hydroxide solution (0.2 ml) and the gas phase was 100% oxygen.

Electron Spin Resonance Spectra were recorded on dry, powdered, melanin samples (35 mg) at room temperature. A Hilger and Watts Microspin X-Band bridge spectrometer with a rectangular cavity operating at 9400 MHz in the H_{012} mode was used. Magnetic field modulation at 100 KHz with phase sensitive detection was employed and first derivative curves were plotted by the pen recorder. g-values were very close to that of diphenylpicrylhydrazyl.

The range of microwave power employed was such that saturation effects were not observed.

Electron Spin Resonance Samples were prepared by the following general procedure.

Oxygen gas was passed for 18 h at 37° through a solution of (±)-3,4-dihydroxyphenylalanine (250 mg) in 0.1 M-phosphate buffer of pH 8.0 (100 ml). Aliquots (50 ml) were taken, 2N-hydrochloric acid (5 ml) was added, the melanin was collected by centrifugation and washed with water (10 ml). The resulting slurry containing *ca.* 60 mg of melanin was used in the experiments described below.

In all experiments a control aliquot (50 ml) was taken and the melanin isolated as above. This sample was not subjected to chemical treatment and was used as a standard for comparison of e.s.r. signal intensities. When "identical signal amplitude" is stated the observed peak heights were equal to ± 5%.

Treatment of Autoxidative Dopa-Melanin with β-NADH. - Distilled water (10 ml) was added to a melanin slurry (*ca.* 60 mg) and the pH of the suspension was adjusted to 8.0 by the dropwise addition of 0.05 N sodium hydroxide solution. A solution of β-NADH (50 mg) in 0.1 M-phosphate buffer, pH 8.0 was added and the suspension was stirred at room temperature for 20 h. The melanin was precipitated by the addition of 2N-hydrochloric acid (5 ml), centrifuged, washed five times with 0.1 N-hydrochloric acid and dried *in vacuo* for 3 days over phosphoric oxide.

Treatment of Autoxidative Dopa-Melanin with Chlorpromazine. - The pH of a solution of chlorpromazine hydrochloride (300 mg) in distilled water (30 ml) was adjusted to 5.0 by the dropwise addition of 10% sodium hydroxide solution. This solution was added to the melanin slurry (ca. 60 mg) and the mixture was stirred at room temperature and pH 5.0 for 20 h. The melanin was precipitated by the addition of 2N-hydrochloric acid (5 ml), centrifuged, washed 3 times with 0.1 N-hydrochloric acid and dried in vacuo over phosphoric oxide.

The amount of chlorpromazine bound to the sample was determined by a u.v. method. The dry melanin (50 mg) was stirred for 2 h with ethanol (50 ml). 2N-hydrochloric acid (2 drops) was added and the suspension was centrifuged. The ethanolic supernatant was decanted, its u.v. spectrum was recorded and compared with that of a chlorpromazine solution of known concentration; $\lambda_{max.}$ (ethanol): 210 (ε = 21,500), 257 (ε = 32,400) and 310 (ε = 4,200) nm. The melanin sample contained chlorpromazine (26 mg).

Treatment of Autoxidative Dopa-Melanin with Potassium Nitrosodisulphonate. - Potassium nitrosodisulphonate was prepared using the procedure of Singh.[30]

To a slurry of the melanin (ca. 60 mg) in distilled water (10 ml) was added a solution of the radical (200 mg) in dilute sodium hydroxide solution, pH 9.0 (10 ml). The suspension was adjusted to pH 9.0 by the dropwise addition of 0.05 M-sodium hydroxide solution and stirred at room temperature for 2 h. The pH of the mixture was maintained close to 9.0 during the course of the reaction by the dropwise addition of the dilute alkali. 2N-hydrochloric acid (6 ml) was added, the melanin collected, washed 4 times with 0.3 N-hydrochloric acid (each 20 ml) and dried in vacuo over phosphoric oxide for 3 days.

Treatment of Autoxidative Dopa-Melanin with NNN'N'-tetramethyl-p-phenylenediamine Bromide. - The radical was prepared by the oxidation of NNN'N'-tetramethyl-p-phenylenediamine with bromine in acetic acid as described by Wurster.[31]

To a melanin slurry (ca. 60 mg) was added a solution of the radical (50 mg) in 50% $^{v}/_{v}$ aqueous acetic acid (20 ml). The suspension was stirred at room temperature for 5 minutes. The melanin was precipitated by the addition of 2N-hydrochloric acid (10 ml), centrifuged, washed twice with 0.1N-hydrochloric acid and dried in vacuo over phosphoric oxide for 3 days.

Treatment of Autoxidative Dopa-Melanin with Ascorbic Acid. - a) To a melanin slurry (ca. 60 mg) in distilled water (10 ml) was added a solution of ascorbic acid (200 mg) in distilled water (30 ml). The mixture was adjusted to pH 9.0 by the dropwise addition of 0.05 N-sodium hydroxide solution and was stirred at room temperature for 21 h. The pH was maintained close to 9.0 during the course of the reaction by the addition of alkali. 2N-hydrochloric acid (5 ml) was added, the melanin was collected, washed 4 times with 0.1 N-hydrochloric acid and dried in vacuo over phosphoric oxide for 3 days.

b) Experiment (a) was repeated with the modification that the reaction mixture was stirred at room temperature and pH 9.0 for 5 days.

c) A solution of ascorbic acid (200 mg) in 20% sodium hydroxide solution (10 ml) was added under nitrogen to a melanin slurry (ca. 60 mg) in 20% sodium hydroxide solution (10 ml). The suspension was stirred under nitrogen at room temperature for 5 h. The melanin was precipitated by the addition of 4N-hydrochloric acid (30 ml), centrifuged, washed 4 times with 0.1 N-hydrochloric acid and dried in vacuo over phosphoric oxide.

Methylation of Autoxidative Dopa-Melanin with Diazomethane. - An excess of ethereal diazomethane (8 ml) was added to a suspension of the finely ground melanin (100 mg) in ether (15 ml) and the mixture was stirred for 12 h. Ether and excess of diazomethane were distilled off. The whole process was repeated twice and the residue was dried in vacuo over phosphoric oxide for 3 days.

Aerial Oxidation of Ascorbic Acid. - a) The pH of a solution of ascorbic acid (200 mg) in distilled water (20 ml) was adjusted to 9.0 by the dropwise addition of 0.05 N-sodium hydroxide solution. The solution was stirred at room temperature for 90 minutes and pH readings were taken at 5 minute intervals.

b) Experiment (a) was repeated using a mixture of autoxidative dopa-melanin (ca. 60 mg) and ascorbic acid (200 mg) in distilled water (20 ml).

Treatment of Autoxidative Dopa-Melanin with Sodium Dithionite. - a) To the melanin slurry (ca. 60 mg) was added a solution of sodium dithionite (200 mg) in distilled water (20 ml) and the pH of the suspehsion was adjusted to 11.0 by the dropwise addition of 0.05 N-sodium hydroxide solution. The mixture was stirred at room temperature for 3 h, acidified with 2N-hydrochloric acid (5 ml) and melanin collected, washed 3 times with 0.1 N-hydrochloric acid (each 15 ml) and dried in vacuo over phosphoric oxide.

b) A solution of sodium dithionite (200 mg) in 20% sodium hydroxide solution (10 ml) was added under nitrogen to the melanin slurry (ca. 60 mg) in 20% sodium hydroxide solution (10 ml). The mixture was stirred under nitrogen for 5 h at room temperature. 4M-hydrochloric acid (30 ml) was added under nitrogen and the light brown coloured melanin was precipitated. The colour darkened appreciably after the melanin was centrifuged and dried in vacuo over phosphoric oxide for 3 days.

Methylation of Autoxidative Dopa-Melanin with Dimethyl Sulphate. - Dimethyl sulphate (2 ml) was added with stirring under nitrogen to the melanin slurry (ca. 120 mg) in 20% sodium hydroxide solution (20 ml). The suspension was stirred under nitrogen at room temperature for 5 h. The melanin was precipitated by the addition of 2 N-hydrochloric acid (60 ml), washed 4 times with 0.05N-hydrochloric acid and dried in vacuo over phosphoric oxide for 3 days.

Treatment of Autoxidative Dopa-Melanin with Potassium Ferricyanide. - A solution of potassium ferricyanide (500 mg) in distilled water (20 ml) was added to the melanin slurry (ca. 60 mg). The suspension was adjusted to pH 10 by the dropwise addition of 0.05 M-sodium hydroxide solution and stirred at room temperature for 20 minutes. The pH of the mixture was maintained close to 10.0 during the course of the reaction by the dropwise addition of the dilute alkali. 2N-hydrochloric acid (10 ml) was added, the melanin collected, washed 5 times with 0.05 N-hydrochloric acid (each 20 ml) and dried in vacuo over phosphoric oxide for 3 days.

Treatment of Autoxidative Dopa-Melanin with Ceric Sulphate. - A solution of ceric sulphate (120 mg) in distilled water (10 ml) was added to the melanin slurry (ca. 60 mg). The suspension, pH 3.0, was stirred at room temperature for 5 minutes. The melanin was centrifuged, washed twice with 0.05 N-sulphuric acid and dried in vacuo over phosphoric oxide for 3 days.

Bound cerous ions were determined by a u.v. method. The dry melanin (50 mg) was stirred for 30 minutes at 60-70° with 3.6 N-sulphuric acid (10 ml). Water (35 ml) was added and the melanin was centrifuged. The acidic supernatant was decanted, its u.v. spectrum was recorded and compared with that of a cerous sulphate solution of known concentration.
$\lambda_{max.}$ (water) : 212 (ε = 280), 223 (ε = 370), 241 (ε = 680) and 253 (ε = 770) nm. The melanin sample contained cerous cations (14 mg).

Treatment of Autoxidative Dopa-Melanin with Zinc Sulphate. - A solution of zinc sulphate (250 mg) in distilled water (10 ml) was added to the melanin slurry (ca. 60 mg) and the mixture was stirred at room temperature for 5 minutes. The melanin was centrifuged, washed twice with distilled water (each 20 ml) and dried in vacuo over phosphoric oxide for 3 days.

Treatment of Autoxidative Dopa-Melanin with Copper Sulphate. - a) A solution of copper sulphate pentahydrate (250 mg) in distilled water (10 ml) was added to the melanin slurry (ca. 60 mg) and the mixture was stirred at room temperature for 5 minutes. The melanin was centrifuged, washed with distilled water (10 ml) and dried in vacuo over phosphoric oxide for 3 days.

b) Experiment (a) was repeated with the modification that the melanin was washed 3 times with 2N-hydrochloric acid (each 10 ml) and then dried in vacuo.

Determination of Cupric Ions Bound by Autoxidative Dopa-Melanin. - a) Two identical melanin slurries were prepared as described p. 12. One batch was dried to constant weight in vacuo over phosphoric oxide and the dry weight of the melanin slurries determined.

To the second melanin slurry (90 mg dry weight of melanin) was added a solution of copper sulphate pentahydrate (50 mg) in distilled water (10 ml) and the mixture was stirred at room temperature for 5 minutes. The melanin was centrifuged, washed with distilled water (10 ml) and boiled with concentrated nitric acid (20 ml) for 3 h. The solution was evaporated to dryness (steam-bath) and the residue dissolved in a mixture of N-sulphuric acid (1 ml) and distilled water (9 ml). 0.1% Sodium diethyldithiocarbamate solution (10 ml) was added and the copper diethyldithiocarbamate complex was extracted 6 times with carbon tetrachloride (each 5 ml). The complex was determined colorimetrically against standard cupric ion solutions.

The melanin contained 0.75 mg of bound cupric ions.

b) Experiment (a) was repeated with the modification that the melanin (70 mg dry weight) was washed 4 times with N-hydrochloric acid (each 10 ml) after treatment with copper sulphate solution.

The melanin contained 0.5 mg of bound cupric ions.

Autoxidation of (±)-3,4-Dihydroxyphenylalanine. - Oxygen uptake was measured manometrically by the Warburg apparatus at 37°. The main compartment of the flask contained (±)-3,4-dihydroxyphenylalanine (0.6 mg) in 0.1M-phosphate buffer (2.8 ml).

When experiments were carried out in the presence of melanin the main compartment contained (±)-3,4-dihydroxyphenylalanine (0.6 mg) together with autoxidative dopa-melanin (0.35 mg) suspended in the 0.1M-phosphate buffer (2.8 ml).

In all experiments the inner cup contained 10% sodium hydroxide solution (0.2 ml) and the gas phase was 100% oxygen.

Preparation of Enzymic Dopa-Melanin. - a) Mushroom tyrosinase (3.7 mg, 10,200 catecholase units) was added to a solution of (±)-3,4-dihydroxyphenylalanine (125 mg) in 0.1 M-phosphate buffer, pH 6.8 (50 ml). Oxygen gas was passed through the solution at 37° for 24 h. The melanin was centrifuged (1,500 g, 5 min), washed twice with distilled water (each 10 ml) and dried in vacuo over phosphoric oxide for 3 days.

Yield 63 mg.

b) Experiment (a) was repeated at 19°. The melanin was not centrifugable (1,500 g, 30 min).

c) Mushroom tyrosinase (4 mg, 11,000 catecholase units) was added to a solution of (±)-3,4-dihydroxyphenylalanine (100 mg) in triply-distilled water (30 ml). Oxygen gas was passed through the solution at 37° for 4 h. The melanin was centrifuged, washed with distilled water (10 ml) and dried in vacuo over phosphoric oxide for 3 days.

Yield 5 mg.

Autoxidation of 3,4-Dihydroxy-6-methylphenylalanine. - Oxygen uptake was measured manometrically in the Warburg apparatus at 37°. The flask contained 3,4-dihydroxy-6-methylphenylalanine (0.6 mg) in 0.1 M-phosphate buffer pH 8.0 (2.8 ml). The inner cup contained 10% sodium hydroxide solution (0.2 ml).

After oxidation was complete the resulting yellow solution was removed from the Warburg flask, diluted with distilled water (10 ml) and its u.v. spectrum was recorded.
$\lambda_{max.}$ (H_2O) : 207 (ε = 7,600), 233 sh. (ε = 3,700), 280 (ε = 1,800) and 340 (ε = 1,500) nm.

LITERATURE CITED

1. G.A. Swan, Fortschritte der Chemie organischer Naturstoffe, 1974, 31, 522.
2. H.S. Raper, Physiol.Rev., 1928, 8, 245.
3. B. Commoner, J. Townsend and G.E. Pake, Nature, 1954, 174, 689.
4. G.K. Fraenkel, J.M. Hirshon and C. Walling, J.Amer.Chem.Soc., 1954, 76, 3606.
5. M.S. Blois, A.B. Zahlan and J.E. Maling, Biophys.J., 1964, 4, 471.
6. M.H. Van Woert, Proc.Soc.Exp.Biol.Med., 1968, 129, 165.
7. H.S. Mason, D.J.E. Ingram and B. Allen, Arch.Biochem.Biophys., 1960, 86, 225.
8. A.M. Potts, Invest.Ophthalmol. 1962, 1, 522.
9. A.M. Potts, Invest.Ophthalmol. 1964, 3, 399.
10. I.S. Forrest, F. Gutmann and H. Keyser, Rev.Agressol., 1966, 7, 147.
11. N.J. Boyd, Ph.D. Thesis, Georgetown University, 1972.
12. J.H. Murib and D.M. Ritter, J.Amer.Chem.Soc., 1952, 74, 399.
13. S.J. Rothman, J.Invest.Dermat., 1942, 5, 67.
14. L. Chauffe, J.J. Windle and M. Friedman, Biophys.J., 1975, 15, 565.
15. R.A. Morton, "Application of Absorption Spectra to Study of Vitamins, Hormones and Coenzymes", Hilger, London, 1942, p. 130.
16. F.H.J. Figge, Proc.Soc.Exp.Biol.Med., 1939, 41, 127.
17. K.C. Das, M.B. Abramson and R. Katzman, J.Neurochem., 1976, 26, 695.
18. E.V. Gan, H.F. Haberman and I.A. Menon, Arch.Biochem.Biophys., 1976, 173, 666.
19. P. Fischer and G. Derouaux, Compt.Rend.Soc.Biol., 1950, 144, 707.
20. T. Sarna and S. Lukiewicz, Folia Histochemica et Cytochemica, 1972, 10, 265.
21. T. Sarna, J.S. Hyde and H.M. Swartz, Science, 1976, 192, 1132.
22. A.I. Vogel, "A Textbook of Quantative Inorganic Analysis", Longman, London, p. 666.
23. H.S. Mason and C.I. Wright, J.Biol.Chem., 1949, 180, 235.
24. F. Binns, R.F. Chapman, N.C. Robson, G.A. Swan and A. Waggott, J.Chem.Soc.(C), 1970, 1128.
25. M.H. Van Woert, K.N. Prasad and D.C. Borg, J.Neurochem., 1967, 14, 707.
26. R.I.T. Cromartie and J. Harley-Mason, Biochem.J., 1957, 66, 713.
27. D.F. Evans, J.Chem.Soc., 1953, 345.
28. J.E. Wertz, D.C. Reitz and F. Dravnieks, in M.S. Blois, "Free Radicals in Biological Systems", Academic Press, New York, p. 183.
29. M. Dixon, "Manometric Methods", Cambridge University Press, 1943.
30. R.P. Singh, Canad.J.Chem., 1966, 44, 1994.
31. C. Wurster, Ber., 1879, 12, 1807.